Protective role of ERCC4 SNP rs1800067 Minor Allele in Gallbladder Carcinogenesis.

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Research Article

Keywords: Single nucleotide polymorphism, Gallbladder Cancer, ERCC4, Nucleotide excision repair, rs1800067, overall survival

Posted Date: March 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-270649/v1

Abstract

Purpose: Gallbladder cancer (GBC) is the most aggressive tumor of the biliary tract. Since DNA damage is one of the common events in GBC, we hypothesized that nucleotide excision repair enzymes may be defective in GBC. We aimed to investigate the association of SNP rs1800067 (G/A) of ERCC4 with the disease predisposition in gallbladder cancer and its prognosis. We have also investigated the expression of ERCC4 in GBC patients and gallstone patients for any possible correlation with the SNP.

Methods: In 350 GBC patients and 300 controls, ERCC4 SNP rs1800067 was genotyped by PCR-RFLP. Semi-quantitative RT-PCR was performed using ERCC4 and internal control β-actin primers in gallstone and tumor biopsy. We adopted the Kaplan-Meier plot and log-rank tests to explore the association of rs1800067 and prognosis of gallbladder cancer patients.

Results: We demonstrate that the minor allele A is less frequent in GBC patients than healthy controls, suggesting the association of GA genotype with decreased risk of GBC. rs1800067 genotypes have significantly differential frequencies relative to clinical parameters. The relative expression of ERCC4 is significantly differentially expressed among early and late stages of tumors. Patients with combined GA+AA genotypes had longer overall survival in patients with early stages of tumors and with chemotherapy.

Conclusion: Our results suggest that minor allele A is significantly associated with reduced risk of gallbladder carcinogenesis. The upregulation of relative mRNA expression of ERCC4 is an early event in the progression of gallbladder cancer.

Introduction

Gallbladder cancer (GBC) is a highly aggressive disease and sixth common cancer of the gastrointestinal tract [1]. GBC is associated with a short survival time after diagnosis. The metastasis of GBC is fast and it easily spreads from one organ to another organ. GBC involves genetic interactions between DNA apoptosis, repair, and inflammatory pathway genes [2]. Many studies have documented that the genes involved in DNA repair and maintenance of genome integrity are critically involved in protecting against mutation that leads to cancer [3]. The major causes of DNA damage in the gallbladder are genomic instability and defective DNA repair pathways [4]. We have attempted to investigate the association of one of the single nucleotide polymorphisms (SNP rs1800067) of ERCC4 (Excision Repair cross-complementation group 4), also known as XPF (Xeroderma Pigmentosum Complementation Group F) which is required for different types of DNA repair with GBC. ERCC4 functions in an irreversible dual incision process during NER by forming a heterodimer complex with ERCC1 and then creating 5' incision to the DNA lesion. It is also responsible for removing UV-C photo-product and bulky adducts from DNA [5]. ERCC4 forms complex with ERCC1 and plays an important role in the maintenance of telomere stability and repair of the inter-strand cross-link. More than a hundred SNPs of ERCC4 have been associated with

several cancers such as bladder, lung, breast, glioma, gastric, head and neck, skin, and colorectal cancers. ERCC4 rs1800067 G/A transition reduces the DNA repair capacity and acts as a predisposing factor in cervical pre-cancer and increased risk of invasive cervical cancer [6]. The expression levels of ERCC4 have been correlated with cancer risk, cancer progression and clinical outcome of multiple human tumors including breast, gastric, pancreas, colorectal, ovarian, cervical, head and neck and osteosarcoma [7–14]. Low expression of ERCC4 is associated with increased risk of cancer due to decreased DNA repair capacity and its higher expression has been shown to reduce the risk of cancer [10, 12]. We hypothesized that ERCC4 rs1800067 G/A that results in a transition from Arg to Gln at codon 415 may alter ERCC4 protein function and thus influence the efficiency of nucleotide excision repair during carcinogenesis. Therefore, we have investigated the association of ERCC4 rs1800067 polymorphism in the population of eastern Uttar Pradesh and western Bihar since this region has a high incidence rate of GBC in India. We have evaluated the influence of rs1800067 with clinical parameters and association with the overall survival. We have also investigated the expression of ERCC4 in GBC patients and gallstone patients for any possible correlation with the SNP.

Materials And Methods

Study population

Peripheral blood and tissue biopsies from the study cohort in this study (belonging to western Bihar and eastern Uttar Pradesh) were collected after informed written consent according to the approved protocol by the Institutional Ethical Committee of Faculty of Science, Banaras Hindu University. Peripheral blood samples were collected from a total of 350 newly diagnosed gallbladder cancer patients (fine-needle aspirated cell cytology (FNAC) and histopathological proven) from the Departments of General Surgery and Surgical Oncology of Sir Sunderlal Hospital, Banaras Hindu University Varanasi, India. The staging of gallbladder cancer was documented according to the American Joint Committee on Cancer. The clinical profile of GBC patients was based on hospital investigation. Peripheral blood from a total of 300 healthy controls were also collected from the general population ethnicity matched to the patients. The inclusion criteria for the control group includes the absence of precancerous lesion, diabetes mellitus, and gallstone proven by ultrasonography. Out of 350 GBC patients, we have also collected 56 gallbladder tumor biopsies from gallbladder cancer patients. Additionally, we also collected 30 gallbladder tissue biopsies from gallstone patients. Patients were followed after two months of the date of diagnosis to their death or last follow up (up to November 30, 2019). Prognostic data such as chemotherapy and overall survival were collected by either hospital visit or telephone interview.

DNA extraction

Blood samples (3-4 ml) of all patients and controls were collected in 0.5 M EDTA (Sigma, USA) vials. Genomic DNA was extracted from both peripheral blood leukocytes using the standard salting-out method. Extracted DNA was quantified using a Nanodrop Analyzer (Thermo Scientific, USA).

Genotyping of the ERCC4 gene polymorphism

The SNP rs1800067 of ERCC4 was selected based on its functional role, reported prevalence of at least 5% for the variant allele and published evidence of its association with cancer. rs1800067 was genotyped by PCR restriction fragment length polymorphism. The sequence of the primer set is as follows: forward 5'TGCCAGAGAGGAGAAAGCAT-3' and reverse 5'- TGGTAGAAGCCCGTTCTTTG-3'. Amplicons of 408 base pairs were analyzed on 2% agarose gel and visualized using UV-transilluminator in gel documentation system (Alpha Innotech, USA). The PCR reaction mixture was digested with PdmI (Thermo Scientific, USA) at 37°C for 16 hours and resolved on 2% agarose gel, visualized and photographed. While Arg allele gives 215 and 193 base pair fragments following PdmI digestion of the PCR product, Gln allele remains undigested fragment of 408 base pairs.

Semi-quantitative reverse transcriptase PCR

Total RNA was isolated from tissue samples using TRizol (Invitrogen, USA). Purified RNA was stored at 80°C. The RNA was quantified by Nanodrop (Thermo Scientific, USA). The first-strand cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (ABI, USA) according to the manufacturer's protocol. Semi-quantitative RT-PCR was performed using ERCC4 and internal control β-actin primers (Supplementary table 1) in gallstone and tumor biopsy samples.

Statistical analysis

The calculated power of our study is >80%. Odds ratios (OR), 95% confidence interval (CI) and p-value for the assessment of associated risk due to genotypes and variant allele of studied polymorphism were calculated by the Epi-Info programme (http://wwwn.cdc.gov/epiinfo/). A p-value < 0.05 is considered as statistically significant. Non-parametric test and one-way ANOVA were performed to analyze relative mRNA ERCC4 expression using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, California USA). One-way ANOVA was used to compare the relative mRNA expression with genotype. We employed the Kaplan-Meier method to draw the overall survival curves of the SNP and used the log-rank test to compare the differences between groups.

Results

A total of 350 GBC patients and 300 controls were enrolled in this study. The clinical characteristics of GBC patients and healthy controls are summarized in Supplementary Table 2. There is a significant difference in age and gender between GBC patients and controls. Interestingly, there is a significant difference (p-value = 0.0013) between early and late age of onset of the disease in GBC patients. Gallstone was present in 60 % of GBC patients and most of the GBC patients presented an advanced stage of cancer (III and IV). Tumor infiltration was observed in 65.4 % and lymph node involvement was observed in 67 % of GBC patients.

Association Of Ercc4 Rs1800067 Polymorphism With Gbc Risks

The genotype and allele frequency of the analyzed SNP along with the resulting odds ratio and the significance level is shown in Table 1. The distribution of the genotype of polymorphism in control is in accordance with the Hardy-Weinberg equilibrium ($p < 0.05$). We found that the G allele is significantly more prevalent and A allele is significantly less prevalent in patients than controls. Our results suggest that the minor allele A is protective. Allele A (OR = 0.50; 95% CI = 0.38–0.67; p-value = < 0.0001) is significantly associated with decreased risk of GBC, comparative genotype frequency distribution in gallbladder cancer patients and control demonstrates that the frequency of the GA (OR = 0.42; 95% CI = 0.23–0.61; p-value = < 0.0001) genotype is significantly associated with decreased risk of GBC. Compared with GA + AA genotypes GG genotype significantly associated with decreased risk of the disease (OR = 0.433; 95% CI = 0.31–0.61; p-value = < 0.0001).

Table 1
Genotype and allele frequency distribution of ERCC4 rs1800067 (G/A) and risk associated with gallbladder cancer

ERCC4	**GBC (N = 350) [n (%)]**	**Controls (N = 300) [n (%)]**	***P value**	**OR (95% CI)**
GG	269 (81.5)	177 (59)	-	1 (Ref.)
GA	69 (19.7)	106 (35.3)	**< 0.0001**	0.42 (0.23–0.61)
AA	12 (3.4)	17 (5.7)	0.05	0.46 (0.22–1.0)
Allele G	607 (86.7)	460 (76.7)	-	(Ref.)
Allele A	93 (13.3)	140 (23.3)	**< 0.0001**	0.50 (0.38–0.67)
Dominant				
GG	269 (76.9)	177 (59)	-	
GA + AA	81 (23.1)	123 (41)	**< 0.0001**	0.433 (0.31–0.61)
Recessive				
GG + AG	338 (96.6)	283 (94.3)	-	
AA	12 (3.4)	17 (5.7)	0.17	0.59 (0.278–1.26)
N = Total number of samples; OR = Odd Ratio; CI = Confidence Interval;				
*Chi-square test.				
p-value < 0.05 is statistically significant and are in bold				

Association Of Ercc4 Rs1800067 Polymorphism With Clinical Parameters

We analyzed the association of ERCC4 rs1800067 with clinical parameters such as age of onset, gender, gallstone status, involvement of lymph node, ascitic status, jaundice, tumor stages, and retroperitoneal or inguinal lymphadenopathy (Table 2). Interestingly, we found that the AA genotype of ERCC4 rs1800067 is significantly more frequent in the early stage of tumors and with the presence of retroperitoneal or inguinal lymphadenopathy than late stages of tumors ($p = 0.027$) and absence of retroperitoneal or inguinal lymphadenopathy ($p = 0.0013$). We did not find a significant difference between the presence or absence of gallstone, involvement of lymph node, distant metastasis, ascites and jaundice for genotypes of ERCC4 rs1800067. GBC patients and controls were stratified according to gender and age of onset. Control and GBC patients below 50 years of age were grouped as early-onset (< 50 years) and more than or equal to 50 years were grouped as late-onset (≥ 50 years) (Table 3). We found that GG, GA, and AA genotypes of ERCC4 rs1800067 (G/A) is significantly different between early and late age of onset between GBC patients and controls. GG and GA genotypes of ERCC4 rs1800067 is significantly different between females and males between GBC patients and controls.

Table 2
Patients characteristics and distribution of ERCC4 rs1800067 (G/A) genotypes

Variables	**Patients**		**Genotypes**		***P-value**
	N (%)	GG	GA	AA	
Gallstone					
Yes	210 (61)	163 (77.6)	41 (19.5)	06 (2.9)	
No	136 (39)	102 (75)	28 (21)	06 (4.5)	0.71
Ascites status					
Yes	69 (20)	56 (81.1)	11 (15.9)	02 (3)	
No	278 (80)	210 (75.5)	58 (20.9)	10 (3.6)	0.61
Retroperitoneal/Inguinal lymphadenopathy					
Yes	68 (19)	52 (76.5)	09 (13.2)	07 (10.3)	
No	280 (81)	215 (77)	60 (21)	05 (2)	**0.0013**
Jaundice status					
Yes	136 (39)	108 (79.4)	22 (16.2)	06 (4.4)	
No	212 (61)	159 (75)	47 (22.2)	06 (2.8)	0.3
Tumor status					
T1 + T2	79 (23)	58 (73.4)	16 (20.3)	05 (6.3)	
T3 + T4	267 (77)	207 (77.5)	53 (19.9)	07 (2.6)	0.29
Lymph-node status					
N0	111 (32)	85 (76.6)	21 (18.9)	05 (4.5)	
N1 + N2	235 (68)	182 (77)	46 (20)	07 (3)	0.77

*Chi-square test.

p-value < 0.05 is statistically significant and are in bold

Variables	Patients		Genotypes		*P-value
Distant metastasis					
M0	232 (67)	173 (74.6)	50 (21.5)	09 (3.9)	
M1	116 (33)	94 (81)	19 (16.4)	03 (2.6)	0.39
Tumor Stages					
I + II	52 (15)	36 (69)	11 (21)	05 (10)	
III + IV	296 (85)	230 (78)	59 (20)	7 (2)	**0.027**
Chemotherapy					
Yes	194 (57)	143 (74)	44 (22)	7 (4)	
No	146 (43)	116 (79)	25 (18)	5 (3)	0.44
*Chi-square test.					
p-value < 0.05 is statistically significant and are in bold					

Table 3
Frequency distribution of ERCC4 rs1800067 (G/A) genotype in GBC patients and controls based on age of onset and gender

Variables	Genotypes			*P-value
	GG	GA	AA	
Age of onset				
Early age of onset				
Cases	102 (77.8)	24 (18.3)	05 (3.8)	
Controls	152 (59.8)	87 (34.3)	15 (5.9)	**0.002**
Late age of onset				
Cases	167 (76.3)	45 (20.5)	7 (3.2)	
Controls	25 (54.3)	19 (41.3)	02 (4.3)	**0.009**
Gender				
Female				
Cases	194 (75.8)	53 (20.7)	9 (2.5)	
Controls	68 (59.6)	39 (34.2)	07 (6.2)	**0.007**
Male				
Cases	75 (79.8)	16 (17)	3 (3.2)	
Controls	109 (58.6)	67 (36)	10 (5.4)	**0.009**
*Chi-square test.				
p-value < 0.05 is statistically significant.				

Survival Analysis

Kaplan–Meier survival curves of follow-up data of available 340 patients to assess the association between the ERCC4 rs1800067 SNPs and survival time of GBC patients did not show a significant difference (p-value = 0.45). We performed additional survival analyses in subgroups of patients stratified by gender, age, TNM stage, jaundice, and chemotherapy. We found that GA + AA genotypes increases the overall survival in patients with early stages of tumors (HR: 0.41; 95% CI: 1.84–0.968; p-value = 0.042) and with chemotherapy (HR: 0.66; 95% CI: 0.44–0.986; p-value = 0.042) (Fig. 1).

Effect Of Ercc4 Rs1800067 Polymorphism On Ercc4 Expression

Our semi-quantitative RT-PCR data did not show significant differential expression of ERCC4 between GBC tumor and gallstone biopsies. The relative mRNA expression profile of ERCC4 showed upregulation in 21% of the tumors, downregulation in 11% of the tumors, and no change in 68 % tumor samples (p-value = 0.26). Collectively, ERCC4 expression showed a significant difference between the early and late stages of tumors (p-value = 0.02) (Fig. 2). Comparison of ERCC4 rs1800067 mRNA expression also did not show a significant difference among genotypes (p-value = 0.34). Our study demonstrates that high expression of ERCC4 does not associated with the overall survival of gallbladder cancer patients (p-value = 0.96).

Discussion

ERCC4 rs1800067, is a nonsynonymous SNP located on exon 8. SNPs located in exonic regions result in amino acid variation in the protein products of genes. To the best of our knowledge, this is the first study to demonstrate the association of ERCC4 SNP rs1800067 with gallbladder cancer and its prognosis. Our study demonstrates that the minor allele A is more frequent in control than patients. A variant allele may become a protective allele or associated with a disease when environment changes [15]. These environmental changes may also be a contributing factor for GBC. Since the geographic distribution of GBC is highly uneven, there may be differences in environmental exposures and regional intrinsic predisposition to carcinogenesis [16]. We demonstrate that genotype GA and allele A of ERCC4 rs1800067 is significantly associated with decreased risk of gallbladder cancer. Our results agree with previous studies linking the allele A with a significantly decreased risk of glioma cancer in the Asian population and lung cancer [17, 18]. Recent studies have shown a significant association of ERCC4 rs1800067 in glioma, gastrointestinal stromal, cervical and meningioma cancer [6, 19–22] but it does not show association with other cancers such as head and neck, thyroid, breast, and osteosarcoma [23–27]. Our results demonstrate that the association of rs1800067 follows the dominant model in gallbladder cancer. Our result similar to previous studies, Wang et al., [28], Ravegnini et al., [21], and Bajpai et al., [6] shows significant association of rs1800067 follows the dominant model in glioma, gastrointestinal stroma, and cervical cancers respectively. Previous studies have reported that genotypes of ERCC4 rs1800067 is not significantly associated with age of onset, gender and tumor stages in GIST [21], larynx cancer [29] and head and neck cancer [23]. However, in the present study, we have found that the genotypes of ERCC4 rs1800067 is significantly different between early and late age of onset of the disease. Also, ERCC4 rs1800067genotypes are significantly different between males and females in GBC patients. AA genotype of ERCC4 rs1800067 is more frequent in the early stages of tumors in GBC patients. These studies suggest that the AA genotype is less aggressive than the GG genotype. Earlier studies have demonstrated that there is no significant association of rs1800067 with overall survival in benign breast cancer, osteosarcoma, and gastrointestinal stromal tumors [21, 26, 30]. Similarly, in the present study, we did not find a significant association of rs1800067 genotypes with overall survival. Our

study demonstrates that A carrier genotypes significantly increases the overall survival in patients with early stages of tumors and with chemotherapy. Discordant to our study, Sun et al. [27] have reported that ERCC4 rs1800067 genotypes did not significantly influence the response to chemotherapy in patients with osteosarcoma. Our result suggests that ERCC4 rs1800067 polymorphism can play an important role in the carcinogenesis of the gallbladder.

Higher expression of ERCC4 has been associated with a longer overall survival (OS) in male patients with colon cancer [10]. Also, ERCC4 has been reported to be a favourable prognostic marker in triple-negative breast cancer [31], esophageal cancer [32], serous ovarian cancer [11], and astrocytoma [33]. We did not find a significant difference in mRNA expression of ERCC4 between gallbladder cancer and control samples. Zhao et al., [11] found that high expression of ERCC4 indicates better survival in ovarian cancer patients. Our study demonstrates that high expression of ERCC4 does not associated with the overall survival of gallbladder cancer patients. Our data demonstrate significant differential ERCC4 expression among early and late stages of tumors. Discordant to our result, Schena et al., [34] have reported that ERCC4 expression did not significantly differentiate between stages of tumors in head and neck squamous cell cancer.

In conclusion, our study demonstrates that the minor allele A is a protective role in gallbladder carcinogenesis in the Indian population. The genotypes GA + AA, being the risk genotype, increases the overall survival in months with early stage of tumor and chemotherapy. Expression profiling results show that the up-regulation of ERCC4 is an early event in the progression of gallbladder cancer.

Declarations

Funding

DST-PURSE, Banaras Hindu University and UGC-UPE Banaras Hindu University for financial assistance to GN, SS; Council for Scientific & Industrial Research, Government of India for Junior Research Fellowship to KA.

Conflicts of interest

Authors declare that there is no conflict of interest.

Availability of data and material*(data transparency)*: N/A

***Code availability*: N/A**

Author's contributions

The experimental work done by KA; Data analysis was done by KA; Sample collection was done by KA and DS; PK and TK provided peripheral blood samples, tumor biopsies, gallstone biopsies and clinical data; The study was planned and supervised by SS and GN; Overall editing was done by SS.

Ethical Approval

The study was approved by Institutional Ethical Committee of Faculty of Science, Banaras Hindu University.

Consent to Participate

The samples were collected after informed written consent of the participants according to the approved protocol by Institutional Ethical Committee of Faculty of Science, Banaras Hindu University.

Consent for publication

All authors have read and give their consent to publish.

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Figures

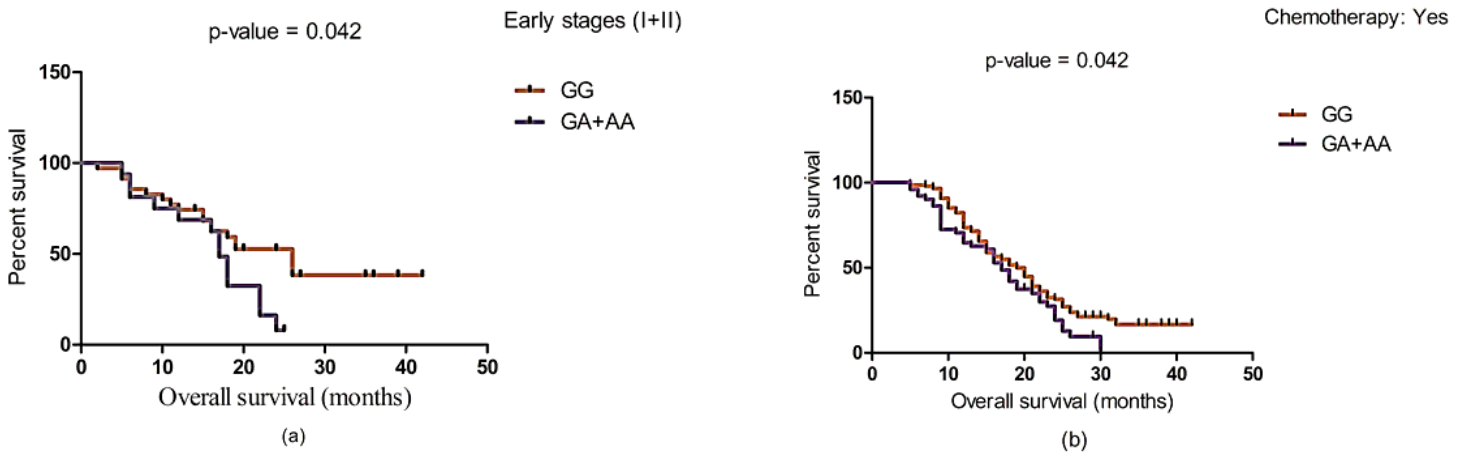

Figure 1

Kaplan–Meier plot of overall survival curves according to ERCC4 rs1800067 G > A genotype in early stage of the tumors (a), and with chemotherapy (b) in gallbladder cancer patients

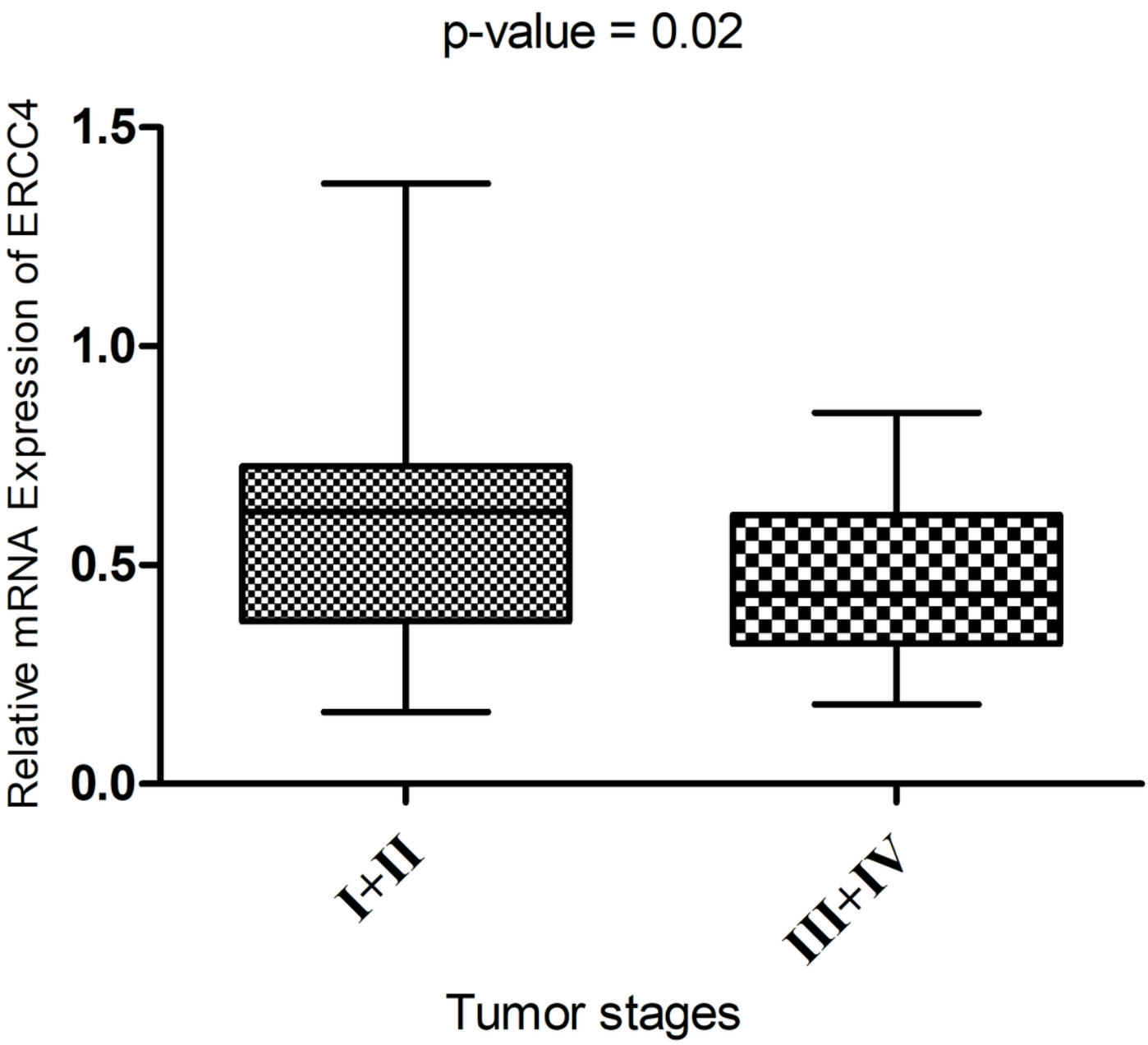

Figure 2

Relative expression of ERCC4 in early and late stages of the tumors

Supplementary Files

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- SupplementaryTable1.docx
- SupplementaryTable2.docx